

Novel X-linked glomerulopathy is associated with a *COL4A5* missense mutation in a non-collagenous interruption

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A novel *COL4A5* mutation causes rapid progression to end-stage renal disease in males, despite the absence of clinical and biopsy findings associated with Alport syndrome. Affected males have proteinuria, variable hematuria, and an early progression to end-stage renal disease. Renal biopsy findings include global and segmental glomerulosclerosis, mesangial hypercellularity and basement membrane immune complex deposition. Exon sequencing of the *COL4A5* locus identified a thymine to guanine transversion at nucleotide 665, resulting in a phenylalanine to cysteine missense mutation at codon 222. The phenylalanine at position 222 is absolutely conserved among vertebrates. This mutation was confirmed in 4 affected males and 4 female obligate carriers, but was absent in 6 asymptomatic male family members and 198 unrelated individuals. Immunostaining for $\alpha 5(\text{IV})$ collagen in renal biopsies from affected males was normal. This mutation, in a non-collagenous interruption associated with severe renal disease, provides evidence for the importance of this structural motif and suggests the range of phenotypes associated with *COL4A5* mutations is more diverse than previously realized. Hence, *COL4A5* mutation analysis should be considered when glomerulonephritis presents in an X-linked inheritance pattern, even with a presentation distinct from Alport syndrome.

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A network formed by self-assembly of $\alpha 3\alpha 4\alpha 5(\text{IV})$ collagen in the glomerular basement membrane (GBM) maintains the structural integrity of the filtration barrier. This function is compromised by certain mutations in the *COL4A3*, *COL4A4*, or *COL4A5* genes, which encode the $\alpha 3\text{--}\alpha 5(\text{IV})$ collagen chains, causing hereditary nephropathies.¹ Mutations in the *COL4A3* or *COL4A4* genes cause autosomal recessive or dominant Alport syndrome.² Mutations in the *COL4A5* gene, located at Xq22, present as X-linked Alport syndrome (XLAS; OMIM no. 301050), which is the most prevalent form of hereditary nephritis.³ The clinical course of XLAS is heterogeneous based on the type of GBM changes, the age of progression to end-stage renal disease (ESRD), and the presence of extrarenal manifestations.^{4,5} Characteristic findings in males include microscopic hematuria in 100%, gross hematuria in 60–70%, proteinuria in 90%, hearing loss in 90%, and ocular abnormalities in 35%.^{5,6} On renal biopsy, extensive thickening and splitting of the GBM in the absence of immune complex (IC) deposition is strongly suggestive of XLAS.⁶ Female heterozygotes for *COL4A5* mutations are less symptomatic than males, but have microscopic hematuria in 95% of cases.⁷

More than 400 mutations in the *COL4A5* gene have been described.⁸ Large case series of XLAS patients have led to the classification of mutations and genotype–phenotype associations.^{4,5,9,10} Major alterations of *COL4A5*—including deletions, frame-shift mutations, donor splice site mutations, and nonsense mutations—result in the absence or severe truncation of the *COL4A5* protein, causing severe XLAS; these patients are characterized by juvenile onset of ESRD and extrarenal features, such as hearing loss and ocular defects.⁵ In contrast, missense mutations of *COL4A5* alter one amino acid residue but produce an otherwise intact *COL4A5* protein, generally causing a less severe clinical presentation, with adult onset of ESRD and fewer extrarenal manifestations.⁵

The goal of this study was to identify the genetic alteration responsible for juvenile-onset ESRD in multiple related males

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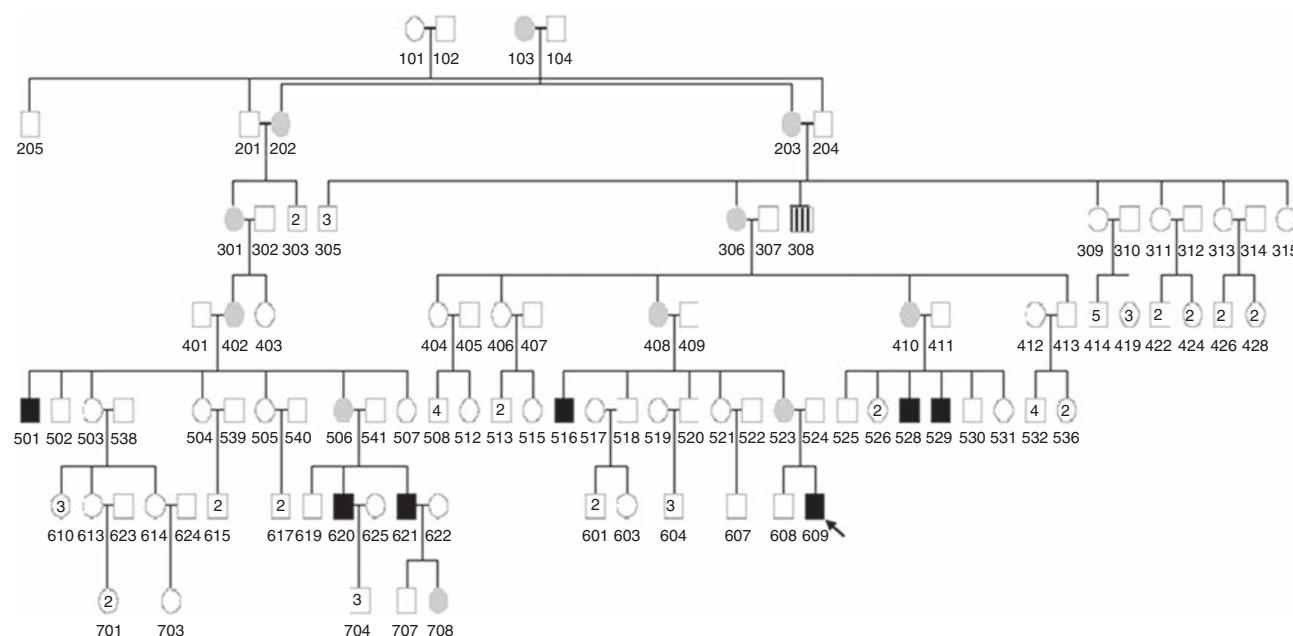


Figure 1 | Extended pedigree. Carriers are indicated by gray circles. Affected males are indicated by black squares. Individual 308 (square with vertical stripes) died of renal failure by history; however, records to determine the precise etiology were not available. The index patient is indicated by an arrow.

of a large pedigree. We report the identification of a novel missense mutation in the *COL4A5* gene strongly associated with kidney disease in this cohort. This mutation occurs within a non-collagenous interruption of the $\alpha 5(\text{IV})$ collagen chain, providing the first direct evidence for the functional importance of this structural motif.

RESULTS

Patients

A pedigree consisting of 117 individuals across 7 generations identified 8 affected males with ESRD and 11 carrier or obligate carrier females (Figure 1). No females were identified with a history of chronic kidney disease. Medical records were available from seven of the affected males; DNA samples were obtained from five affected males, four carrier females and six unaffected males. The absence of glomerular disease in unaffected males was confirmed with a dipstick urinalysis with no more than trace blood or protein.

Presentation and clinical course

The clinical presentation of affected males (Table 1) was notable for proteinuria, hypertension, and renal dysfunction with variable hematuria. The two males with no hematuria at presentation did not develop persistent hematuria during progression to ESRD. Two of the patients had documented hypoalbuminemia and four patients had nephrotic range proteinuria. Two affected males had normal audiograms at presentation, and none reported hearing loss or hearing aid requirements at the time of consent or had documentation of hearing loss in the medical records. One patient had visual changes, determined to be secondary to pseudotumor

cerebri. No patients had documented anterior lenticonus or esophageal leiomyomatosis. One patient had linear immunoglobulin G (IgG) deposits on a renal allograft biopsy. One of the evaluated female carriers had trace hematuria, and two out of three (67%) manifested 100 mg/dl of proteinuria.

Renal biopsy findings

Records from nine renal biopsies performed on seven patients were reviewed (Table 2). Tissue from five biopsies was available for review. Light microscopy findings included global and segmental sclerosis (Figure 2a) and mesangial expansion (Figure 2b). No foam cells were identified. Immunofluorescence was consistently positive for IgM (granular) in the GBM, with weak (1+) intensity. No biopsy was positive for IgA. Electron microscopy revealed scattered electron dense deposits in the GBM (Figure 3). Podocyte foot process effacement was present in areas with and without basement membrane dense deposits (Figure 3). Three of the NCH biopsies had small subepithelial lucent areas suggestive for resolved deposits. Only one patient had splitting of the GBM. However, the splitting was confined to areas of dense deposits or lucent areas, indicating that it was most likely a secondary change. No patient had diffuse thinning of the GBM.

COL4A5 sequencing

Sequencing of the coding region for *COL4A5* identified a thymine to guanine (T>G) transversion at nucleotide 665 (T665G) in exon 12 of the index patient, resulting in a single phenylalanine to cysteine (Phe→Cys) amino acid substitution at codon 222 (p.F222C). Exon 12 was subsequently amplified by PCR from genomic DNA of additional family

Table 1 | Clinical presentation and course

Patient	How diagnosed	BP and physical examination	Initial serum labs	Initial urine laboratory data	Treatment	Age at diagnosis/onset of ESRD (year)	Long term outcome
501	Data not available.	Data not available.	Data not available.	Data not available.	Data not available.	Unknown/17	Two renal transplants. Linear fluorescence for IgG noted on renal allograft biopsy. This patient died at age 26 years.
516	Proteinuria was identified during the annual primary care evaluation.	BP, 132/70. No edema.	ANA, negative. Cr, 0.4 mg/dl. C3, 130 mg/dl.	2+ Protein. Small blood. 20–30 rbcs/hpf.	Prednisone and leukeran.	9/16	A renal transplant has been functioning for the last 24 years.
529	Proteinuria was identified during the annual primary care evaluation.	Data not available.	Data not available.	Data not available.	Data not available.	13/16	The patient has had three renal transplants.
528	Proteinuria was identified during the annual primary care evaluation.	BP, 120/90. No edema.	ANA, negative. Cr, 1.1 mg/dl. C3, 86 mg/dl.	4+ Protein. 5–10 rbcs/hpf.	Data not available.	9/13	A renal transplant has been functioning for the last 27 years.
620	A renal evaluation was performed secondary to renal disease in his brother.	BP, 170/110. Pretibial and periorbital edema.	Albumin, 2.5 g/dl. Cr, 2.2 mg/dl. C3, 95 mg/dl. C4, 14 mg/dl.	4+ Protein. Trace blood.	Ace inhibition.	17/22	A renal transplant has been functioning for the last 19 years.
621	The patient was noted to be hypertensive during an evaluation for headaches.	BP, 150/100. No edema.	Albumin, 2.2 g/dl. ANA, negative. Cr, 4.3 mg/dl. C3, 141 mg/dl. C4, 28 mg/dl.	4+ Protein. No blood. 5.4 mg/kg/hr of protein.	Ace inhibition and imuran.	13/14	A renal transplant has been functioning for the last 21 years.
609	The patient was evaluated in the emergency department with nausea, vomiting and fatigue.	BP, 220/150. No edema.	ANA, negative. Cr, 11.4 mg/dl. C3, 130 mg/dl. C4, 31 mg/dl.	4+ Protein. <1 rbc/hpf. Protein: Cr, 15.2 mg/mg.	Renal replacement therapy.	10/10	The patient has been transplanted within the last year.

Abbreviations: Ace, angiotensin-converting enzyme; ANA, anti-nuclear antibody; BP, blood pressure; Cr, creatinine; hpf, high-power field; IgG, immunoglobulin G; rbc, red blood corpuscle.

The clinical presentation and course of seven related male patients are summarized. Numbers designate the location of each patient in the extended pedigree (Figure 1). Serum creatinine in mg/dl may be converted to $\mu\text{mol/l}$ by multiplying by 88.4. Blood pressures are listed as mm Hg.

members and sequenced (Supplementary Figure S1 online). We confirmed the presence of the mutation in another 4 affected males; in addition, one mutant allele was present in 4 carrier females and absent in 6 asymptomatic males (Table 3), as well as 198 unrelated individuals. These findings suggest that the p.F222C mutation is causative of the X-linked glomerulopathy in this family.

Renal survival

All affected males developed ESRD. The age of 50% renal survival was 16 years in males with the p.F222C mutation compared with 28 years, 26 years, and 22 years in historical controls with *COL4A5* splice site mutations, missense mutations, and large rearrangements, nonsense and deletion/insertion mutations, respectively (Figure 4).^{9,10}

Expression of collagen IV chains

Immunohistochemistry (IHC) was performed on available diagnostic renal biopsy tissue from four affected males, as

well as positive and negative controls. Results were consistent, with all four biopsies demonstrating positive (but slightly lighter than positive control) staining for $\alpha 1(\text{IV})$ collagen and $\alpha 3(\text{IV})$ collagen, and positive staining for $\alpha 5(\text{IV})$ collagen. Representative results are presented in Figure 2c.

DISCUSSION

We report a series of related male patients whose presentation and clinical course included proteinuria with variable hematuria; mesangial hypercellularity with IC deposition on renal biopsy; and rapid progression to ESRD. We identified a novel T>G transversion at nucleotide 665 in *COL4A5*, which segregated in affected males and female carriers but was absent in unaffected males and 198 unrelated individuals, indicating that it is likely causative. The amino acid residue altered by this mutation, Phe-222, occurs within one of the 22 breaks that interrupt the Gly-X-Y repeats comprising the collagenous domain of the $\alpha 5(\text{IV})$ collagen chain.⁴ The amino acid sequence of this interruption,

Table 2 | Renal biopsy findings

Patient	501	516	516	528	529 ^a	529	620	621	609
Age at biopsy (years)	Unknown	9	14	10	13	16	17	13	9
Biopsy reviewed at NCH		+	+				+	+	+
<i>Light microscopy</i>									
Mesangial expansion	+	+	+	+			+	+	+
FSGS		+	+	+			+	+	+
Global sclerosis (affected/total)		+	+	+		+	+	+	+
		1/15	2/8			All	9/21	3/6	16/19
Interstitial fibrosis							+	+	+
Inflammation							+		+
<i>Immunofluorescence</i>									
IgA									
IgM		+	+	+			+	+	+
		gbm	gbm				gbm mes	gbm mes	gbm
IgG		+		+					
		gbm							
C3		+	+	+			+	+	
		gbm	mes				gbm mes	gbm	
<i>Electron microscopy</i>									
Electron dense deposits		+	+	+			+	+	
		s-ep	s-en				s-en	s-en	
		i-me	i-me				s-ep	s-ep	
		s-en	mes				mes	mes	
							i-me		
GBM splitting		+ ^b	+ ^b						
Lucent areas			+				+	+	+
			s-ep					s-ep	s-ep

Abbreviations: FSGS, focal segmental glomerulosclerosis; gbm, glomerular basement membrane; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; i-me, intramembranous; mes, mesangium; NCH, Nationwide Children's Hospital; s-en, subendothelial; s-ep, subepithelial.

Shaded cells indicate when data was not available.

The renal biopsy findings of seven related male patients are summarized. In certain cases, serial biopsies were available. Patients are designated according to their location in the family pedigree (Figure 1). The presence of a finding is indicated by a '+' symbol in the appropriate box.

^aSpecific details of light microscopy not available, but findings reported as 'normal'.

^bThe splitting was confined to areas of deposits or lucent areas.

GLNFQG, has been phylogenetically conserved from fish to mammals suggesting an important structural and functional role for this interruption (Figure 5).

Breaks in Gly-X-Y repeats introduce bends in the collagen triple helix, create flexible sites, and may form binding sites.^{11,12} G4G interrupts (Gly-X1-X2-X3-X4-Gly) and G1G (Gly-X1-Gly) interrupts are the most common in all collagen IV chains.¹³ Peptide studies show that G4G interrupts have an ordered structure, forming a pseudo-triple helix, in which hydrophobic interactions among residues at the X3 position—like Phe-222—replace glycine packing in the triple helix.^{14,15} Substitution of Phe-222 is predicted to disrupt these hydrophobic interactions, altering the normal structure of this G4G interruption. Development of severe kidney disease in patients with the p.F222C mutation highlights the functional importance of Phe-222. Notably, this is the first collagen IV missense mutation causing severe kidney disease that occurs within a Gly-X-Y interruption.

Although *COL4A5* mutations typically cause XLAS, the affected males in this study have clinical and pathological features inconsistent with the classic presentation of XLAS. Microscopic hematuria occurred in only three out of five (60%) of the affected males and one out of three (33%) of the

carrier females in our series, compared with 100% of males and 95% of females for XLAS.^{5,6} As the initial urinalysis in affected males without hematuria was performed late in the clinical course, it remains possible that hematuria is more prominent in an earlier phase of the disease. Male patients without hematuria concurrently had nephrotic range proteinuria and elevated serum creatinine. The median renal survival age was 15 years, compared with 25 years for XLAS.⁵ Despite the severity of the nephritis, there were no documented extrarenal complications typically associated with severe XLAS. However, such extrarenal manifestations cannot be conclusively ruled out without formal audiologic and ophthalmologic evaluation. Mesangial hypercellularity and segmental sclerosis may be identified on renal biopsies of XLAS patients, but are accompanied by more characteristic findings.¹⁶ Although the basement membranes were thickened, the absence of alternate thickening and thinning of the GBM, known as 'basket weaving' and splitting of the GBM (with the exception of one limited area in a single case that was suggestive of a secondary change) is unusual for XLAS.¹⁶

Basement membrane electron dense deposits and positive immunofluorescence are generally thought to be inconsistent with XLAS, though exceptions have been reported in AS

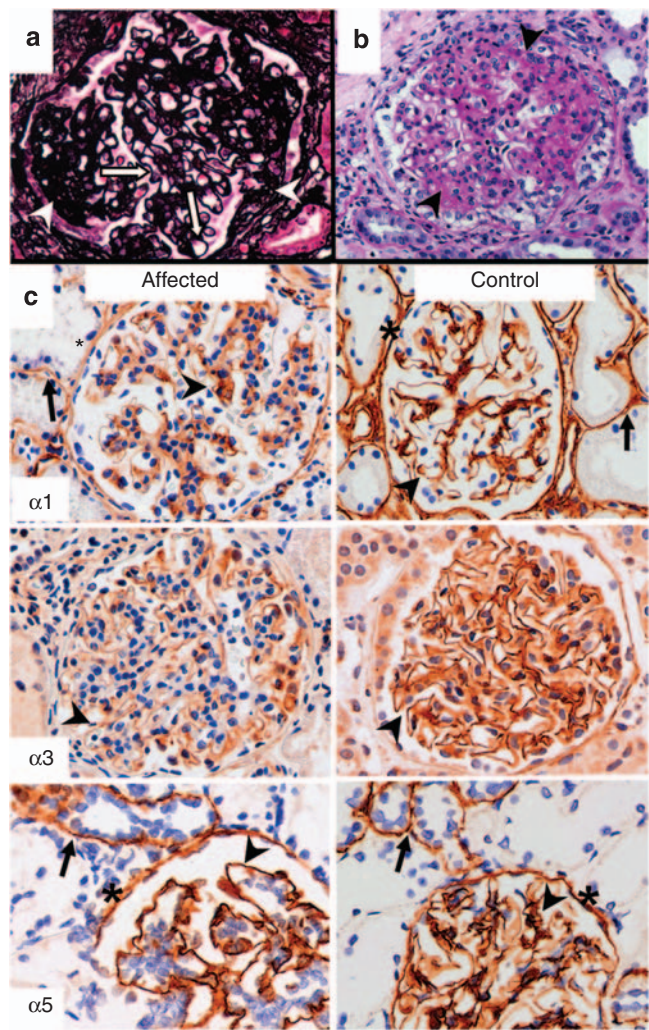


Figure 2 | Light microscopy and immunohistochemistry findings in the renal biopsy. (a) Jones silver staining demonstrated segmental sclerosis (arrowheads). No splitting, spikes or lucencies were evident in the glomerular basement membrane (GBM) (arrows). (b) Periodic acid-Schiff staining demonstrates mesangioproliferative glomerulonephritis with increased mesangial matrix and mesangial cellularity (arrowheads). Magnification $\times 400$. (c) Staining for $\alpha 1$ (IV), $\alpha 3$ (IV), and $\alpha 5$ (IV) collagen was found in the GBM (arrowheads), although $\alpha 3$ (IV) staining was slightly diminished in affecteds. Positive staining was present in the tubular basement membranes (arrows); this staining was diffuse for $\alpha 1$ (IV) collagen and focal for $\alpha 3$ (not shown) and $\alpha 5$ chains of type IV collagen. $\alpha 1$ (IV) and $\alpha 5$ (IV) collagen was also expressed in the basement membranes of Bowman's capsule (asterisks).

patients.^{17,18} In addition, IC deposition has been reported in Alport syndrome patients with a coexisting glomerulopathy, that is, dual glomerulopathy.^{19,20} The mechanism of IC deposition in patients with *COL4A5* mutations is not known. We were unable to detect the presence of circulating anti-GBM antibodies in serum from the index patient by indirect immunofluorescence (D-BB, WL, data not shown); however, material from additional affected individuals was unavailable, and it remains conceivable that serum factors (such as

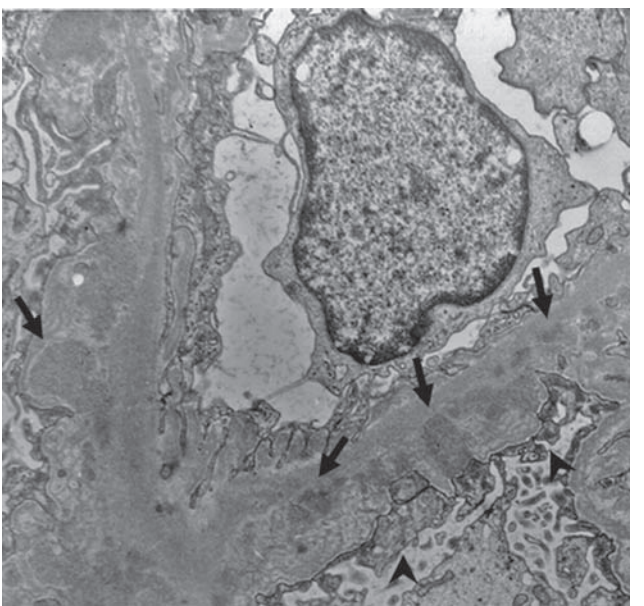


Figure 3 | Electron microscopy findings in the renal biopsy. Electron microscopy results are presented with electron dense immune complex (arrows) deposition. Effacement of the podocyte foot processes is present (arrowheads).

Table 3 | COL4A5 genotyping and screening UA results

Subject	Phenotype	Blood	Protein	Genotype
409	UN	Negative	Trace	
413	UN	Negative	Trace	WT
502	UN	Negative	Trace	WT
541	UN	Negative	Trace	
518	UN	Negative	Trace	WT
520	UN	Negative	Trace	WT
525	UN	Negative	Trace	WT
619	UN			WT
408	C	Negative	2+	
410	C	Trace	2+	WT/T665G
506	C	Negative	Trace	WT/T665G
523	C			WT/T665G
708	C			WT/T665G
516	A			T665G
528	A			T665G
620	A			T665G
621	A			T665G
609	A			T665G

Abbreviations: A, affected; C, carrier; UN, unaffected; WT, wild type. Subjects are designated according to their location in the extended pedigree (Figure 1) and grouped according to their clinical phenotype, as UN, C, or A. When available, the results of dipstick urinalysis for blood and protein are listed (negative). Results of *COL4A5* nucleotide 665 genotyping are listed as WT or the T665G transversion. Shaded cells indicate when data was not available.

autoantibodies) could account for the glomerular deposition of ICs. Alternatively, structural changes in the collagen IV network of the GBM may lead to IC trapping independent of antigen recognition, which in turn contributes to glomerular damage. The presence of IC raises the question of whether antibody trapping leads to complement activation and the potential role of complement in glomerular damage in

affected patients. There was no evidence of hypocomplementemia in all examined individuals at presentation (Table 1), arguing against complement activation. Immunofluorescence demonstrated positive C3 in five out of six biopsies examined, with GBM localization in three out of five cases (Table 2). The presence of C3 deposition in the GBM has been reported in XLAS, particularly in segmental lesions with progression of disease.²¹ Whether this represents non-specific trapping of C3 along with antibodies such as IgG and IgM or complement activation is unclear at this time.

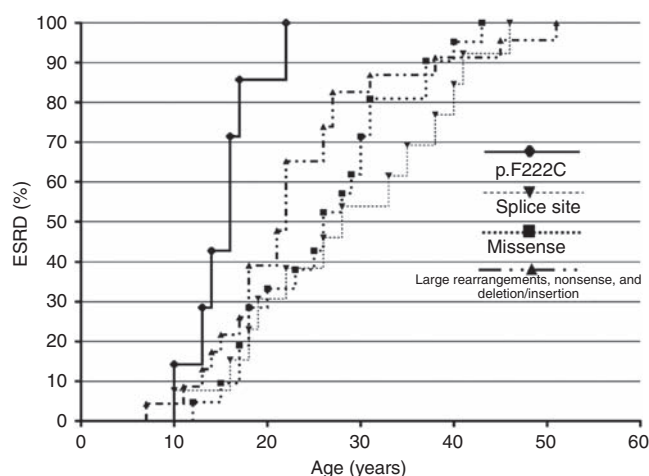


Figure 4 | Renal survival curves. Patients with the p.F222C mutation ($n = 7$) progressed to ESRD at a more rapid rate than historical controls with splice site mutations ($n = 13$), missense mutations ($n = 21$), and large rearrangements, nonsense and deletion/insertion mutations ($n = 23$) reported by Martin *et al.*³⁰ and Gross *et al.*⁹ Differences between patients with the p.F222C mutation and patients with other types of mutations were highly significant ($P < 0.001$).

The positive staining for $\alpha 5(\text{IV})$ and $\alpha 3(\text{IV})$ collagen in the renal biopsies of the affected patients indicates that the p.F222C mutation does not interfere with the assembly of $\alpha 3\alpha 4\alpha 5(\text{IV})$ collagen. This is not surprising, because the specific assembly of $\alpha 3\alpha 4\alpha 5(\text{IV})$ molecules and networks is driven by molecular recognition sites within the non-collagenous (NC1) domains at the carboxyl end of each collagen IV chain.^{22,23} The most frequently detected missense mutations map to conserved glycine residues within the Gly-X-Y repeats comprising the collagenous domain of $\alpha 5(\text{IV})$ collagen.^{5,24} Mutations of these glycine residues introduce an abnormal break in the collagen triple helix, altering the structure and reducing the stability of $\alpha 3\alpha 4\alpha 5(\text{IV})$ molecules. As a result, the affected patients develop comparatively milder forms of XLAS.⁵ More severe forms of XLAS, with juvenile onset of ESRD, are caused by COL4A5 mutations such as large deletions, nonsense mutations, or splice site donor mutations that yield a truncated $\alpha 5(\text{IV})$ chain lacking the NC1 domain.⁵ Because these mutations prevent the assembly of $\alpha 3\alpha 4\alpha 5(\text{IV})$ heterotrimers, they cause the absence of $\alpha 3$, $\alpha 4$ and $\alpha 5(\text{IV})$ collagen from the GBM, resulting in a more severe phenotype.²⁵ In our series of patients, severe kidney disease is not due to the impaired deposition of $\alpha 3\alpha 4\alpha 5(\text{IV})$ collagen in the GBM, but may reflect a specific functional impairment of this network caused by the p.F222C mutation.

Although the precise mechanism whereby the p.F222C mutation results in kidney disease remains to be determined, some contributing factors may be inferred. As discussed, the pseudo-triple helical structure of G4G interruptions is stabilized by interactions among hydrophobic residues at the third position, such as Phe-222. Although cysteine is hydrophobic, it is not a conservative substitution for phenylalanine because of its smaller size and propensity to

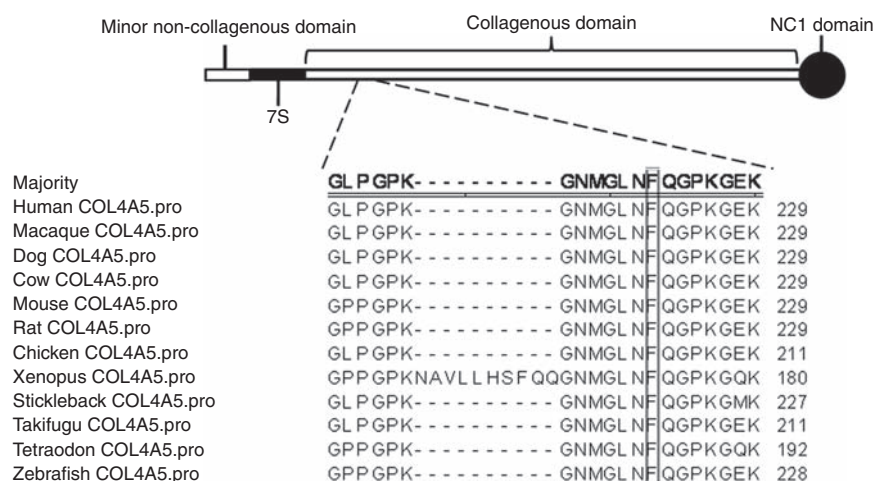


Figure 5 | Phylogenetic conservation of the Phe-222 residue. Type 4 collagens consist of a minor non-collagenous domain, a minor collagenous (7S) domain, a major collagenous domain containing 15–20 noncollagenous interruptions and a carboxyl terminal non-collagenous (NC1) domain.^{32,33} The area of the non-collagenous interruption affected by the p.F222C mutation is represented by the dashed lines. The alignment of the predicted COL4A5 protein sequences from 12 species reveals the complete conservation of the Phe-222 residue in vertebrates.

form disulfide bonds. The collagenous triple helix of $\alpha3\alpha4\alpha5(\text{IV})$ molecules is characterized by numerous intra- and intermolecular disulfide bridges.²⁶ The collagenous domains of $\alpha3(\text{IV})$ and $\alpha4(\text{IV})$ contain 7 and 15 cysteine residues, respectively, whereas that of $\alpha5(\text{IV})$ (residues 42–1456) contains only 3 cysteine residues at positions 451, 481, and 484. Therefore, introduction of a new cysteine at position 222 in the $\alpha5(\text{IV})$ chain can have a detrimental effect on the structure and function of the $\alpha3\alpha4\alpha5(\text{IV})$ collagen network by interfering with the formation of the normal disulfide bonds and/or by introducing a new disulfide bridge at an undesirable position. Cysteine substitutions in other collagenopathies appear to dramatically alter the phenotype. Defects of *COL1A1* or *COL1A2* typically involve substitution of a glycine in the Gly-X-Y repeat of the triple helix domain, resulting in osteogenesis imperfecta.²⁷ Interestingly, when cysteine is substituted for arginine in the triple repeat, the phenotype changes to that of Ehlers-Danlos with a high propensity for arterial rupture and minimal bone involvement.²⁸ Functional studies indicate the cysteine causes abnormal formation of disulfide bridges, which can result in either premature degradation of the protein or delayed secretion and formation of abnormal collagen fibrils in the extracellular matrix (depending on the location of the mutation). We speculate a similar phenomenon may be occurring with this *COL4A5* mutation, with secretion of an intact but abnormal protein.

Because of random X chromosome inactivation, females carriers with XLAS have variable expression of the mutant $\alpha5(\text{IV})$ collagen chain—even within a given glomerulus—and as many as 25% develop severe renal disease.²⁹ Of the five carrier females enrolled in our study, all denied a history of abnormal renal function upon interview, and renal function tests were not available. Yet two out of the three (67%) carrier females evaluated by urine dipstick did manifest 2+ proteinuria, suggestive of GBM abnormalities. Whether GBM abnormalities do occur and result in subclinical disease could not be further assessed because of the absence of kidney biopsy tissue from carrier females.

In summary, we have described a set of patients with X-linked glomerulopathy without characteristic findings of XLAS by clinical history or renal biopsy findings who progressed to ESRD at an early age. To our knowledge, this is the first report to associate IC deposition with a specific mutation in the *COL4A5* gene. Our findings emphasize that the detection of IC deposition should not exclude the diagnosis of XLAS, nor should it automatically implicate the p.F222C mutation described in this study. In addition, this is the first *COL4A5* mutation associated with rapid progression to ESRD, in patients otherwise lacking the characteristic clinical and biopsy findings associated with XLAS. Finally, this is the first *COL4A5* missense mutation that occurs within a non-collagenous interrupt of $\alpha5(\text{IV})$ collagen. Testing for *COL4A5* mutations should be a consideration when glomerulopathy presents with an X-linked inheritance pattern, regardless of the consistency of the presentation with XLAS. Future studies should identify the structural and functional differences between mutant and wild-type *COL4A5* protein

and determine if the p.F222C mutation is also present in unrelated patients with similar biopsy findings.

METHODS

Patient selection

This study was approved by the Nationwide Children's Hospital Institutional Review Board. Participants were enrolled with informed consent. A pedigree was constructed based on interviews and historical records. Dipstick urinalysis was performed by presumed unaffected male as well as female family members. Controls comprised race-matched individuals of North European ancestry from Centre de' Etude du Polymorphisme Humain (Paris, France) families (120 individuals) obtained from the Coriell Cell Repository (Camden, NJ), and from central Ohio (78 individuals).

DNA isolation and *COL4A5* sequencing

DNA isolated from peripheral blood of the index patient (no. 609) was submitted for exon sequencing of *COL4A5* (Athena Diagnostics, Worcester, MA). For sequencing of *COL4A5* exon 12, DNA was isolated from saliva (Oragene, Kanata, Ontario, Canada). PCR Primers flanking exon 12 of *COL4A5* were: 5'-TATCTTTTATTTGGTGTG GA-3' and 5'-TCAAGAAGCAAGAGAAAAGAA-3'. Conditions were: 10 min at 94 °C; then 36 cycles of 94 °C for 30 s, 59.3 °C for 30 s, 72 °C for 45 s; then 72 °C for 10 min, then 4 °C. PCR reactions were purified and sequenced bidirectionally.

Probability of ESRD curve analysis

Graphs for the age of ESRD in the affected males with the p.F222C mutation and historical controls^{9,30} were generated with GraphPad Prism v5.0b (GraphPad Software, La Jolla, CA) by the Kaplan-Meier method. Differences between curves were analyzed for significance with the Log-rank (Mantel-Cox) Test.³¹

IHC analyses

As the frozen sections were not available, expression of collagen IV chains was analyzed by IHC using 3- μm thick sections of formalin-fixed, paraffin-embedded renal needle biopsies. IHC was automated on the BondMax IHC system (Leica Microsystems, Bannockburn, IL). For antigen retrieval, sections were treated with proteinase K for 10 min at 37 °C. Mab-1 and Mab-5 (Alpco Diagnostics, Salem, NH) were used for staining $\alpha1(\text{IV})$ and $\alpha5(\text{IV})$ collagen, respectively. Mab-5D6, used for detection of $\alpha3(\text{IV})$ collagen, is a murine IgG2a monoclonal antibody raised against recombinant human $\alpha3\text{NC1}$ monomers (Molecular Recognition Shared Facility, Vanderbilt University). The specificity of mAb 5D6 was verified by indirect ELISA using recombinant NC1 monomers and by immunofluorescence staining of normal human kidney (D-BB, unpublished observations). Staining with mAb 5D6 or Mab-5 was performed after treatment with acid urea (Alpco Diagnostics). Positive-control sections were obtained from a normal autopsy kidney. Negative controls were obtained by substituting the primary antibody with a Universal Negative Control Serum (cat no. NC49BL, Biocare Medical, Concord, CA).

Evaluation of phylogenetic sequence conservation

Protein sequence alignments to evaluate phylogenetic sequence conservation were performed with MegAlign (DNASTAR, Madison, WI), using the Clustal W method.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. COL4A5 Genotyping.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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